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ALKYLTHIO ACETIC ACIDS (3-THIA FATTY ACIDS)—A NEW GROUP OF NON-β-OXIDIZABLE PEROXISOME-INDUCING FATTY ACID ANALOGUES—II

DOSE–RESPONSE STUDIES ON HEPATIC PEROXISOMAL- AND MITOCHONDRIAL CHANGES AND LONG-CHAIN FATTY ACID METABOLIZING ENZYMES IN RATS

ROLF KRISTIAN BERGE,*† ASLE AARSLAND,* HARALD KRYVI,‡ JON BREMER§ and NIELS AARSAETHER*

*Laboratory of Clinical Biochemistry, University of Bergen, Haukeland Sykehus, N-5021 Bergen; ‡Zoological Laboratory, University of Bergen, Bergen; and §Institute of Medical Biochemistry, University of Oslo, P.O. Box 1112, Blindern, Oslo 3, Norway

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Abstract—The activity of key enzymes involved in oxidation and esterification of long-chain fatty acids was investigated after male Wistar rats were treated with different doses of sulfur substituted fatty acid analogues, 1,10-bis(carboxymethylthiodecane) (BCMTD, non-β-oxidizable and non-ω-oxidizable), 1-mono(carboxymethylthiotetradecane) (CMTTD, trivial name, alkylthio acetic acid, non-β-oxidizable) and 1-mono(carboxyethylthiotetradecane) (CETTD trivial name, alkylthio propionic acid, β-oxidizable). The sulfur substituted dicarboxylic acid and the alkylthio acetic acid induced in a dose-dependent manner the mitochondrial, microsomal and especially the peroxisomal palmitoyl-CoA synthetase activity, the mitochondrial and cytosolic palmitoyl-CoA hydrolase activity, the mitochondrial and especially the microsomal glycerophosphate acyltransferase activity and the peroxisomal β-oxidation, especially revealed in the microsomal fraction. Morphometric analysis of randomly selected hepatocytes revealed that BCMTD and CMTTD treatment increased the number, size and volume fraction of peroxisomes and mitochondria. Thus, the observed changes in the specific activity of fatty acid metabolizing enzymes with multiple subcellular localization can partly be explained as an effect of changes in the s-values of the organelles as proliferation of mitochondria and peroxisomes occurred. The most striking effect of the alkylthio propionic acid was the formation of numerous fat droplets in the liver cells and enhancement of the hepatic triglyceride level. This was in contrast to BCMTD treatment which decreased the hepatic triglyceride content. In conclusion, the results provide evidence that administration of non-β-oxidizable fatty acid analogues had much higher *in vivo* potency in inducing hepatomegaly and key enzymes involved in fatty acid metabolism, including proliferation of peroxisomes and mitochondria than is exhibited in the β-oxidizable, alkylthio propionic acid. Moreover, the dicarboxylic acid was apparently three to six times more potent than the alkylthio acetic acid in inducing peroxisomal β-oxidation and peroxisome proliferation when considered on a μmol/day basis. As palmitic acid and hexadecanedioic acid only marginally affected these hepatic responses, it is conceivable that the potency of the selected compounds as proliferators of peroxisomes and inducers of the associated enzymes depends on their accessibility for β-oxidation.

A number of changes are observed on hepatic fatty acid metabolism and morphology notably at the level of peroxisomes, when peroxisome proliferating compounds such as hypolipidemic drugs [1–4], phthalate ester plasticizers [5–7], leukotriene antagonists [8, 9] and high fat diets [10–13] are administered to rats *in vivo*. A considerable proliferation of small peroxisomes is observed, with lower s-values‖ and an increased capacity of β-oxidation of long-chain fatty acids [2, 3]. These treatments do not only induce peroxisomal enzymes but also extraperoxisomal enzymes, including microsomal cytochrome P-452 [14], cytosolic palmitoyl-CoA hydrolase [3–5, 15], clofibroyl-CoA hydrolase [16] and fatty acid metabolites as CoA [4, 17–19] and carnitine [20]. Starvation [21] and diabetes [22] also induce the activity of peroxisomal β-oxidation. Thus, this multitude of responses may be an adaptive change initiated to correct a disturbance in lipid metabolism.

Various mechanisms have been proposed to explain the induction of peroxisomal β-oxidation. Whether enzyme induction may be regulated by substrate supply [4, 17] or the hypolipidemic drugs themselves are converted into unmetabolizable CoA thioesters [17, 23–25] which subsequently bind to a recognition site, should be considered. The possibility should also be considered that inhibition of mitochondrial fatty acid oxidation at the level of carnitine palmitoyltransferase I i.e. formation of acylcarnitines of hypolipidemic drugs may be involved in the induction of peroxisomal enzymes [9, 26–28].

The level of long-chain acyl-CoA is likely to be determined by the balance between the rate of delivery of fatty acids to the liver, the rate of activation

† To whom all corresponence should be addressed.

‖ Abbreviation: s, the average sedimentation coefficient for a group of particles at 4°.

Table 1. Structural formulae, names and abbreviations of sulfur substituted fatty acid analogues and ordinary fatty acids

Structure of compound	Systematic names and abbreviations	Trivial names
$HOOC—CH_2(CH_2)_{13}—S—CH_2COOH$	1,10-Bis(carboxymethylthio decane) (BCMTD) (non-β-oxidizable, non-ω-oxidizable)	Alkylthio dicarboxylic acid
$CH_3—(CH_2)_{13}—S—CH_2COOH$	1-(Carboxymethylthio)tetradecane (CMTD) (non-β-oxidizable)	Alkylthio acetic acid
$CH_3—(CH_2)_{13}—S—CH_2—CH_2COOH$	1-(Carboxyethylthio)tetradecane (CETTD) (β-oxidizable)	Alkylthio propionic acid
$CH_3—(CH_2)_{13}—CH_2—COOH$	Palmitic acid (PMA) (β-oxidizable)	
$HOOC—CH_2—(CH_2)_{12}—CH_2COOH$	Hexadecanedioic acid (HDDA) (β-oxidizable)	

of fatty acids to acyl-CoA and the rate of utilization for oxidation, esterification and hydrolysis. Adaptive changes in the activities of the enzymes involved in these pathways may reflect alterations in the metabolic flux through the corresponding pathways.

The studies here described were done to compare the hepatic dose–response of key-enzymes involved in oxidation and esterification of fatty acids in rats to β-oxidizable and non-β-oxidizable sulfur substituted fatty acid analogues (Table 1). Particular interest has been focused on which of the enzyme systems change in specific activity and subcellular distribution. High fat diets containing fatty acids which are poorly metabolizable, alter the peroxisome- and especially the mitochondria morphology [3, 12]. Therefore, as the synthesized sulfur substituted derivatives can be considered as fatty acid analogues, (non-β-oxidizable as well as β-oxidizable), morphometric analysis of hepatocytes were performed with special reference to peroxisomes and mitochondria.

MATERIALS AND METHODS

Chemicals and drugs. [1-^{14}C]Palmitic acid, [1-^{14}C]palmitoyl-CoA, L-[^{14}C]carnitine and [1-^{14}C]-glycerol-3-phosphate, purchased from New England Nuclear (Boston, MA), were mixed with unlabelled palmitic acid, palmitoyl-CoA, L-carnitine and glycero-3-phosphate (Sigma Chemical Co., MO) to give a specificity of 1500–1800 cpm/nmol. Hexadecanedioic acid (HDDA) was obtained from Aldrich-Chemie (Steinheim, F.R.G.). 1,10-Bis(carboxymethylthio) decane (BCMTD), 1-(carboxymethylthio) tetradecane (CMTTD) and 1-(carboxyethylthio) tetradecane (CETTD) were prepared as earlier described [24, 25]. All other chemicals were obtained from common commercial sources and were of reagent grade.

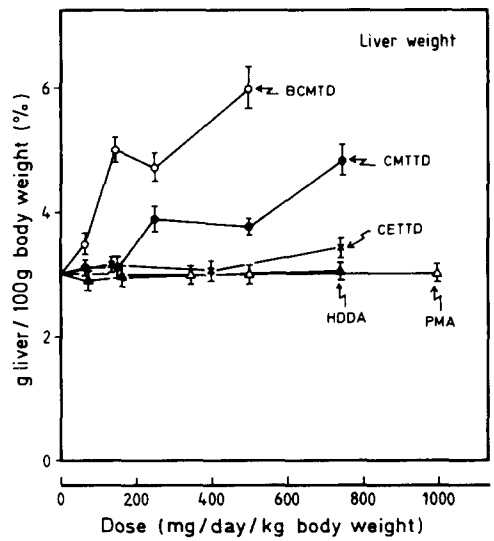

Fig. 1. Effect of increasing doses of the sulfur substituted dicarboxylic acid (BCMTD) (○—○), alkylthio acetic acid (CMTTD) (●—●), alkylthio propionic acid (CETTD) (×—×), palmitic acid (PMA) (△—△) and hexadecanedioic acid (HDDA) (▲—▲) on rat liver weight.

Animals and treatments. Male Wistar rats from Möllegaard Breeding Laboratory (Ejby, Denmark), weighing 170–180 g, were housed individually in metal wire cages in a room maintained 12 hr light–dark cycles and a constant temperature of 20 ± 3°. The animals were acclimatized for at least one week under these conditions before the start of the experiment. BCMTD, CMTTD, CETTD, HDDA and palmitic acid were suspended in 0.5% sodium carboxymethyl cellulose (CMS). The individual agents were administered by gastric intubation in a volume of 1 ml once a day for 5 days and the animals were killed at the start of the sixth day after 12 hr of starvation. The animals were separately treated with the fatty acids and the doses were: BCMTD, 75, 150, 250 and 500 mg/day/kg body wt; CMTTD, 75, 150, 250, 500 and 750 mg/day/kg body wt; CETTD, 150, 400 and 800 mg/day/kg body wt; HDDA, 75, 150 and 750 mg/day/kg body wt; palmitic acid, 350, 500 and 1000 mg/day/kg body wt: The control animal groups recieved only CMS. All animals had free access to water and food.

The body weights were measured daily. At the end of the experiments, the fasted rats were weighed, exsanguinated to obtain blood samples and the livers were removed and immediately chilled on ice and weighed.

Preparation of total homogenate and the different subcellular fractions. The livers from individual rats were homogenized in ice-cold sucrose-medium (0.25 M sucrose in 10 mM Hepes buffer, pH 7.4 and 1 mM EDTA) and the resulting nuclear plus postnuclear fraction was used as the total homogenate.

For further analytical differential centrifugation experiments post-nuclear fractions from three animals were pooled, and a mitochondrial-enriched

Table 2. Effect of sulfur substituted fatty acid analogues and normal fatty acids on serum and hepatic lipids of rats

Compounds	Feeding dose (mg/day/kg body wt)	Serum lipids		Hepatic lipids	
		Triglyceride (μmol/ml)	Cholesterol (μmol/ml)	Triglyceride (nmol/g liver)	Cholesterol (nmol/g liver)
Control	—	0.95 ± 0.17	1.73 ± 0.32	6.79 ± 0.22	9.79 ± 0.35
BCMTD	150	0.64 ± 0.09*	0.86 ± 0.20*	2.93 ± 0.33*	9.94 ± 0.26
CMTTD	150	0.66 ± 0.10*	0.90 ± 0.13*	6.42 ± 0.59	9.37 ± 0.08
CETTD	150	0.93 ± 0.80*	1.32 ± 0.88**	23.6 ± 2.20*	10.03 ± 0.31
Palmitic acid	150	1.20 ± 0.10	1.74 ± 0.17	6.75 ± 0.24	9.84 ± 0.24
Hexadecanedioic acid	150	0.94 ± 0.18	1.75 ± 0.21	6.30 ± 0.28	9.74 ± 0.33

The tabulated values represent means ± SD of twelve control and six animals in each experimental group. Statistical significance compared to control, *$P < 0.01$, **$P > 0.05$.

fraction (M), peroxisome-enriched fraction (L), microsomal fraction (P) and cytosolic fraction (S) were isolated [3, 24].

The variation in the response from animal to animal was estimated separately for selected enzymes in the group of control animals.

Enzyme assays. The subcellular marker enzymes were determined as earlier described [13, 24, 25]. Protein was assayed by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

The enzymatic activity of palmitoyl-CoA synthetase (EC 6.2.1.3), carnitine palmitoyltransferase (EC 2.3.1.21), glycerophosphate acyltransferase (EC 2.3.1.15), palmitoyl-CoA hydrolase (EC 3.1.2.2) and palmitoyl-CoA dependent dehydrogenase (usually termed peroxisomal β-oxidation) were determined as earlier described [30, 31]. Results are expressed as mean ± SD. Statistical analysis was by Student's *t*-test. $P > 0.05$ was taken to be statistically insignificant.

Morphological methods. Morphometric analysis was carried out as earlier described [2, 12].

RESULTS

Effect of sulfur substituted fatty acid analogues and normal fatty acids

Liver weight. Diet intakes of all animal groups were comparable and the increases in body weights were similar for the feeding groups.

The relative liver weight increased in a dose-dependent manner to the non-β-oxidizable sulfur substituted dicarboxylic acid (BCMTD) feeding resulting in a two-fold increase at a dose of 500 mg/day/kg body wt (Fig. 1). No hepatomegaly was observed after the alkylthio acetic acid (CMTTD) administration up to a dose of 150 mg/day/kg body wt. Higher doses, however, gradually increased the liver weight and in the two highest dosage groups (500 and 750 mg/day/kg body wt) the liver weight was increased 1.3- and 1.6-fold, respectively (Fig. 1). Increased liver weight after the β-oxidizable alkylthio propionic acid (CETTD) administration to rats was only observed in the highest dosage group (750 mg/day/kg body wt). No hepatomegaly resulted after palmitic acid and hexadecanedioic acid feeding.

Hepatic and serum lipids. The synthesized sulfur substituted fatty acid analogues decreased the serum cholesterol and triglyceride levels. The order of potency with respect to reduction of serum lipids was BCMTD = CMTTD > CETTD (Table 2). The sulfur substituted hypolipidemic drugs also affected the hepatic lipids, especially the triglyceride level (Table 2). BCMTD, which is not oxidizable at all decreased the hepatic triglyceride content about 50% whereas alkylthio propionic acid, which is theoretically oxidizable, increased the hepatic triglylceride level about 3.5-fold (Table 2). Alkylthio acetic acid which can be ω-oxidized and subsequently β-oxidized, did not change the hepatic triglyceride at a dose of 150 mg/day/kg body wt. The hepatic cholesterol level was not changed by these sulfur substituted fatty acid analogues. Palmitic acid and HDDA did not change neither the triglyceride nor the cholesterol level in rat liver (Table 2).

Fatty acid metabolizing enzymes and subcellular fractionation studies. The distribution of protein and marker enzymes for mitochondria, peroxisomes, lysosomes and microsomes was for all groups of animals essentially similar to our previous findings of rat liver homogenates [3, 13, 16]. The liver protein was not significantly changed. Recovery of protein and enzyme activities were essentially the same for all dosage groups and in the range 95 to 106% (data not shown).

A high purity of the mitochondrial fraction, and in particular of the microsomal fraction was found, as judged by the distribution of marker enzymes. Based on the total activities, the amounts of glutamate dehydrogenase, succinate phenazine methosulfate oxidoreductase, catalase, urate oxidase and lactate dehydrogenase suggest a 3–7% contamination of mitochondria, peroxisomes and cytosol in the microsomal fraction. The amounts of rotenone-insensitive NADPH cytochrome *c* reductase and lactate dehydrogenase suggest a 1–8% contamination of microsomes and cytosolic proteins in the mitochondrial fraction. The amounts of catalase and urate oxidase suggest a 10–15% contamination of peroxisomes in the mitochondrial fraction. Based on total activities, the amounts of NADPH–cytochrome *c* reductase and glutamate dehydrogenase suggest a 1–12% contamination with mitochondrial and microsomal marker enzymes in the peroxisome-enriched fraction.

The activities of selected fatty acid metabolizing enzymes were measured in total liver homogenates and cellular fractions after administrating the animals

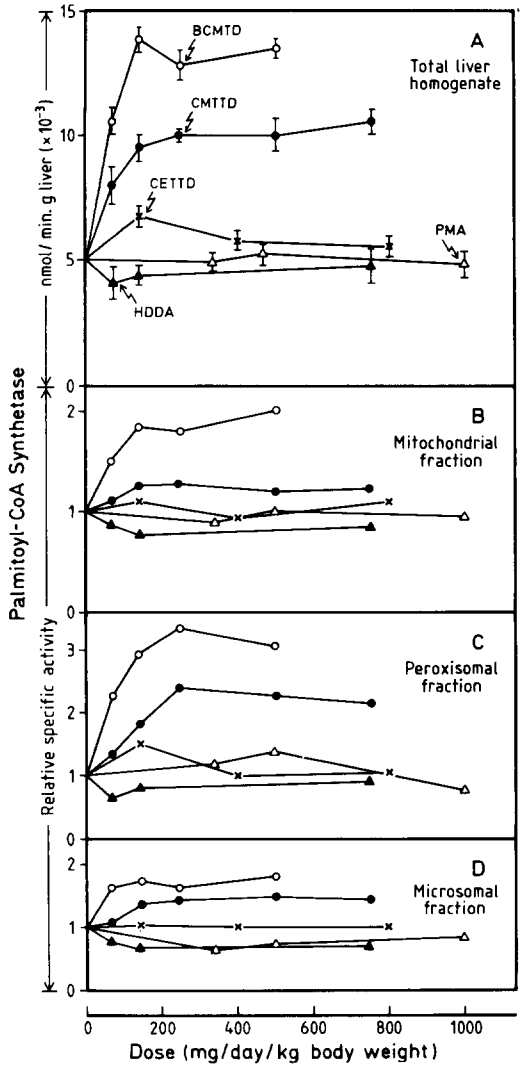

Fig. 2. Effect of sulfur substituted fatty acid analogues (BCMTD, CMTTD, CETTD) and normal fatty acids (PMA, HDDA) on palmitoyl-CoA synthetase activity in total liver homogenates (A), mitochondrial fraction (B), peroxisomal-enriched fraction (C) and microsomal fraction (D). For symbols see Fig. 1. The specific activities are calculated relative to those of caroxymethyl cellulose-fed controls = 1.

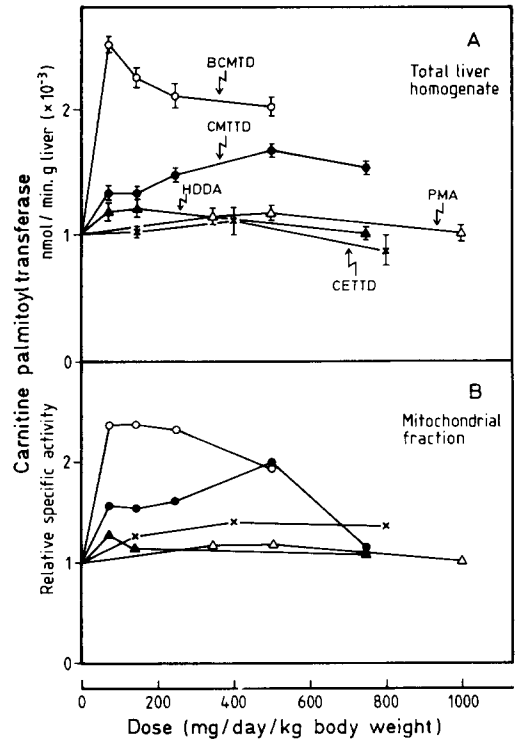

Fig. 3. Dose–response study of carnitine palmitoyltransferase activity in total liver homogenates (A) and mitochondrial fraction (B) by BCMTD, CMTTD, CETTD, PMA and HDDA administration. For symbols and calculation see Figs. 1 and 2.

different amounts of fatty acid analogues and normal fatty acids for five days.

Palmitoyl-CoA synthetase activity. The palmitoyl-CoA synthetase activity of the total liver homogenates (Fig. 2A), mitochondrial fraction (Fig. 2B) and microsomal fraction (Fig. 2D) increased to its maximum value at a dose of 150 mg/day/kg body wt BCMTD. At that dose the activity in the corresponding fractions was increased 2.7-, 1.9- and 1.7-fold, respectively. The palmitoyl-CoA synthetase activity, however, in the total liver homogenates (Fig. 2A), mitochondrial fraction (Fig. 2B) and microsomal fraction (Fig. 2D) of alkylthio acetic acid (CMTTD) administered rats reached a maximal level at a dose 250 mg/day/kg body wt. Furthermore, the increases in enzyme activities were lower in magnitude to the changes observed after BCMTD feeding. Noteworthy, among the cellular fractions of BCMTD and CMTTD dosage groups the highest stimulation of palmitoyl-CoA synthetase activity was observed in the isolated peroxisome-enriched fraction (Fig. 2C). At a dose of 250 mg/day/kg body wt, the peroxisomal palmitoyl-CoA synthetase activity was enhanced 3.4-fold after BCMTD feeding and 2.5-fold after CMTTD administration (Fig. 2B).

The palmitoyl-CoA synthetase activity of the total liver homogenates of alkylthio propionic acid (CETTD) fed rats only increased 1.3-fold (Fig. 2A). Higher doses gradually normalized the enzyme activity. A similar tendency was observed both in the mitochondrial- and peroxisomal fractions (Fig. 2B and C) whereas the microsomal palmitoyl-CoA synthetase activity remained unchanged (Fig. 2D). Marginal effects of the palmitoyl-CoA synthetase activity were seen in both the liver homogenates (Fig. 2A) as well as in the cellular fractions (Fig. 2) of palmitic acid treated animals. HDDA administration at the two lowest dosage groups slightly, but significantly, decreased the palmitoyl-CoA synthetase activity in total liver homogenates (Fig. 2A) and all cellular fractions (Fig. 2B, C and D).

Carnitine palmitoyltransferase activity. A significant increase (2.6-fold) of the carnitine palmitoyltransferase activity in the total liver homogenate was found with the lowest dose of the sulfur substituted dicarboxylic acid (BCMTD) (75 mg/day/kg

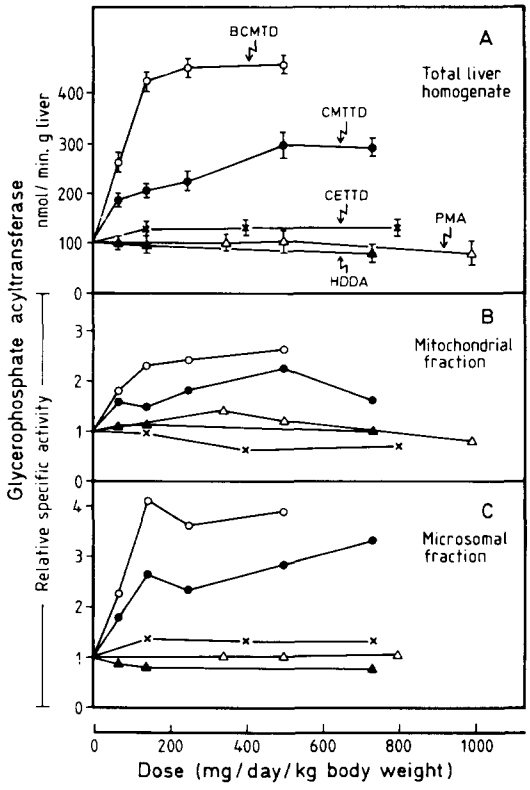

Fig. 4. Dose-dependent changes of glycerophosphate acyltransferase activity in total liver homogenates (A), mitochondrial fraction (B) and microsomal fraction (C) after feeding rats increasing concentrations of BCMTD, CMTTD, CETTD, PMA and HDDA. For symbols and calculation see Figs. 1 and 2.

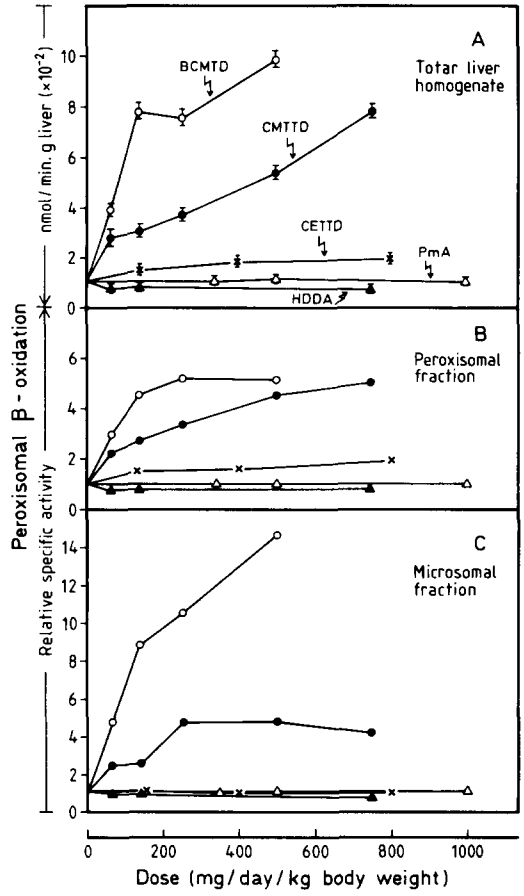

Fig. 5. Effect of increasing doses of BCMTD (○—○), CMTTD (●—●), CETTD (×—×), PMA (△—△) and HDDA (▲—▲) on peroxisomal β-oxidation in total liver homogenates (A), peroxisome-enriched fraction (B) and microsomal fraction (C). See Fig. 2 for calculation.

body wt) (Fig. 3A). Higher doses tended to normalize the carnitine palmitoyltransferase activity. A similar dose-pattern of carnitine palmitoyltransferase activity was also revealed in the mitochondrial fraction (Fig. 3B). In contrast to BCMTD feeding, the carnitine palmitoyltransferase activity was steadily increasing both in the total liver homogenate (Fig. 3A) as well as in the mitochondrial fraction (Fig. 3B) up to a dose of 500 mg/day/kg body wt of alkylthio acetic acid. At that dose, the mitochondrial enzyme activity was increased two-fold (Fig. 3B). Alkylthio propionic acid administration also increased the carnitine palmitoyltransferase activity with increasing doses, especially revealed in the mitochondrial fraction (Fig. 3B). At a dose of 400 mg/day/kg body wt CETTD, the mitochondrial enzyme activity increased 1.4-fold.

The carnitine palmitoyltransferase activity was slightly affected after administration of palmitic acid whereas a slight, but significantly increased enzyme activity (1.2-fold) was observed at the lowest dose of HDDA administration (Fig. 3). Higher doses normalized the carnitine palmitoyltransferase activity.

Glycerophosphate acyltransferase activity. The dose-dependency of glycerophosphate acyltransferase activity was also measured. BCMTD was considerably more potent than CMTTD in inducing stimulation of the enzyme activity in all investigated cellular fractions (Fig. 4). Furthermore, maximal induction of glycerophosphate acyltransferase activity occurred at different dose levels for the two inducers. With a dose of 150 mg/day/kg body wt dicarboxylic acid, a maximal increase in enzyme activity was reached in total liver homogenates (4.4-fold), whereas a dose of 500 mg/day/kg body wt alkylthio acetic acid gave a maximal stimulation of glycerophosphate acyltransferase resulting in a 3-fold increase (Fig. 4A). It was also interesting to note that the dose-pattern of the mitochondrial and microsomal glycerophosphate acyltransferase differed subsequent to BCMTD- and CMTTD-feeding (Fig. 4B and C).

In BCMTD-fed animals the mitochondrial glycerophosphate acyltransferase was gradually increased with increasing doses, and in the highest dosage group (500 mg/day/kg body wt) a 2.6-fold increase was reached (Fig. 4B). In contrast to the activity of the mitochondrial enzyme, the microsomal glycerophosphate acyltransferase was maximally increased over 4-fold at a dose of 150 mg/day/kg body wt (Fig. 4C).

Increasing doses of CMTTD gradually increased the glycerophosphate acyltransferase both in the

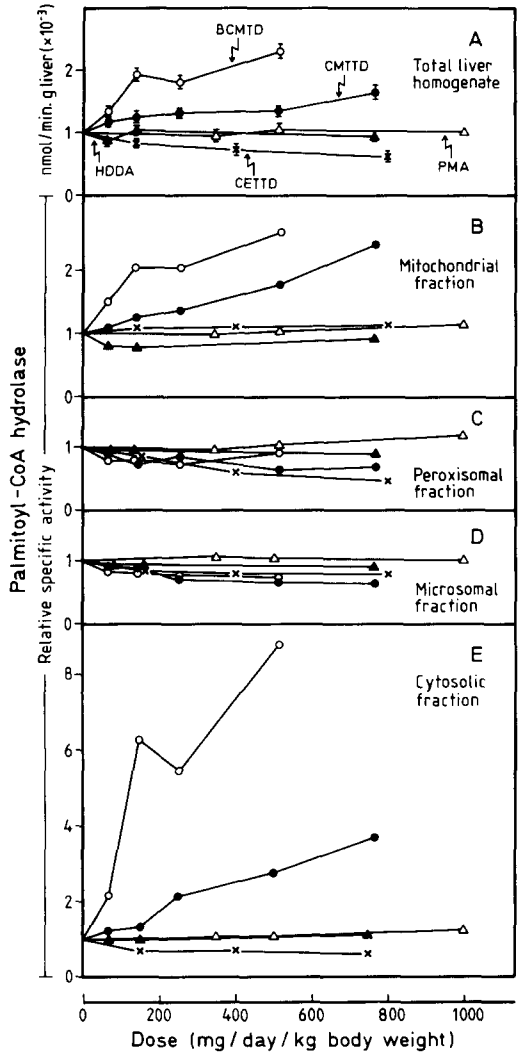

Fig. 6. Dose-dependent changes of palmitoyl-CoA hydrolase activities in total liver homogenates (A), mitochondrial fraction (B), peroxisomal fraction (C), microsomal fraction (D) and cytosolic fraction (E) by BCMTD, CMTTD, CETTD, PMA and HDDA. For symbols and calculation see Figs. 1 and 2.

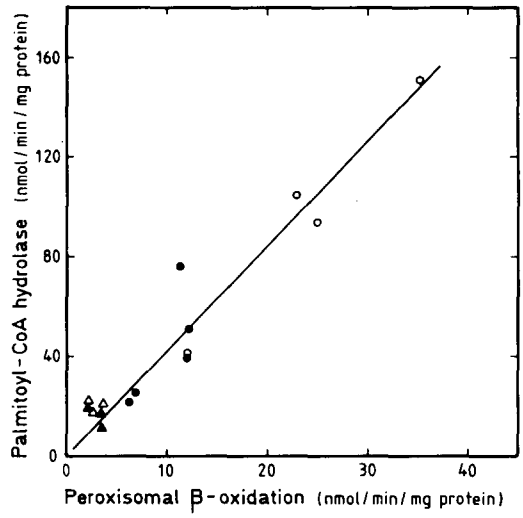

Fig. 7. Correlation of the peroxisomal β-oxidation in the microsomal fraction (Fig. 5C) and cytosolic palmitoyl-CoA hydrolase activity (Fig. 6E) after feeding rats increasing doses of BCMTD (○—○), CMTTD (●—●), CETTD (△—△) and carboxymethyl cellulose-fed control rats (▲—▲).

microsomal- and mitochondrial fractions. Again, the highest stimulating effect was observed in the microsomal fraction (Fig. 4). The lowest dose applied of alkylthio propionic acid (150 mg/day/kg body wt) caused the highest increase in glycerophosphate acyltransferase activity both in total liver homogenates (Fig. 4A) and microsomal fraction (Fig. 4C). The stimulation, however, was marginally; only 1.4-fold increase for the microsomal glycerophosphate acyltransferase activity. The mitochondrial glycerophosphate acyltransferase activity, however, revealed a decrease in the CETTD dosage groups (Fig. 4B). At a dose of 400 mg/day/kg body wt the mitochondrial enzyme activity was decreased about 40%.

Up to a dose of 500 mg/day/kg body wt of palmitic acid the mitochondrial glycerophosphate acyltransferase activity was slightly increased whereas the microsomal enzyme activity remained unchanged (Fig. 4B and C). HDDA administration changed the glycerophosphate acyltransferase activity *vice versa*—the mitochondrial activity was marginally affected whereas the mitochondrial enzyme activity decreased (Fig. 4B and C).

Peroxisomal β-oxidation. Peroxisomal β-oxidation activity also showed a dose-dependent induction following administration of the sulfur substituted fatty acid analogues (Fig. 5). Again the maximal enhancement of enzyme activity in total liver homogenates (Fig. 5A) as well as in cellular fraction (Fig. 5B and C) occurred at different dose levels for the three inducers. It was of interest to note that the increase in peroxisomal β-oxidation (5-fold) in the peroxisomal-enriched fraction (Fig. 5B) was similar in magnitude in animals fed BCMTD and CMTTD whereas the activity both in the total liver homogenates (Fig. 5A) and microsomal fraction (Fig. 5C) was enhanced more after feeding the sulfur substituted dicarboxylic acid than alkylthio acetic acid. Furthermore, it should be noted that the enhancement of peroxisomal β-oxidation in the peroxisomal-enriched fraction was less pronounced than in the total liver homogenates and especially in the microsomal fraction (Fig. 5). In the last fraction, at equal doses, the peroxisomal β-oxidation in the BCMTD dosage group increased about 14-fold whereas a 5-fold increase resulted in the CMTTD dosage group (Fig. 5C).

Administration of alkylthio propionic acid (CETTD) resulted in increased peroxisomal β-oxidation activity (2-fold) both in the total liver homogenates (Fig. 5A) and in the peroxisome-enriched fraction (Fig. 5B). Notably, no increased enzyme activity was found in the microsomal fraction (Fig. 5C). The peroxisomal β-oxidation of the palmitic acid fed animals remained unchanged whereas HDDA administration tended to decrease the

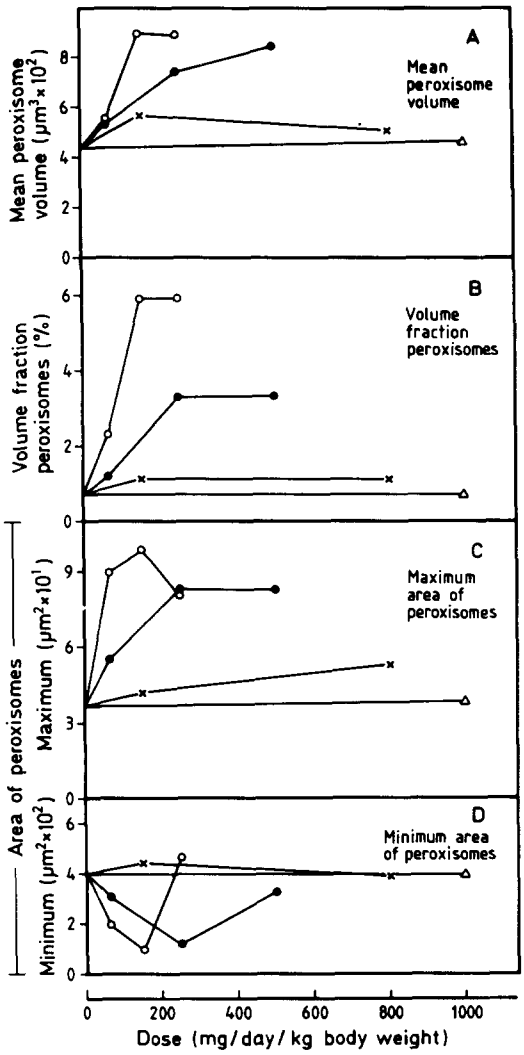

Fig. 8. Morphometric parameters of peroxisomes in hepatocytes of rats fed increasing doses of BCMTD (○—○), CMTTD (●—●), CETTD (×—×) and PMA (△—△).

activity of the enzyme system in all fractions (Fig. 5).

Palmitoyl-CoA hydrolase activity. The palmitoyl-CoA hydrolase activity in total liver homogenates increased in a dose-dependent manner subsequent to both CMTTD and BCMTD feeding (Fig. 6A). In contrast, in conjunction with increasing doses of CETTD, a gradual decrease in enzyme activity was observed. A 40% reduction was obtained at the highest dose (Fig. 6A). The palmitoyl-CoA hydrolase activity remained unchanged in the HDDA- and palmitic acid-treated animals.

The palmitoyl-CoA hydrolase activity in the cellular fractions was affected differently with increasing doses of the experimental compounds. Mitochondrial (Fig. 6B) and cytosolic (Fig. 6E) palmitoyl-CoA hydrolase activity was increased in a dose-related fashion in the CMTTD- and BCMTD-fed animals. The cytosolic enzyme, however, was induced to a greater extent than the mitochondrial enzyme up to the dose of 500 mg/day/kg body wt (BCMTD, 8.5-fold compared to 2.5-fold; CMTTD, 3-fold compared to 1.8-fold). In addition, the maximum induction of these parameters occurs at different dose-levels for the two inducers. BCMTD appears to be much more potent than CMTTD considered at equal molar basis. In contrast to CMTTD and BCMTD administration, alkylthio propionic acid feeding tended to increase the mitochondrial palmitoyl-CoA hydrolase activity whereas (Fig. 6B) the cytoslic enzyme activity was decreased (Fig. 6E). The cytosolic palmitoyl-CoA hydrolase activity remained unchanged after palmitic acid and HDDA feeding (FIg. 6E). However, palmitic acid administration tended to increase the mitochondrial enzyme activity at the highest dosage group whereas HDDA tended to decrease the mitochondrial palmitoyl-CoA hydrolase activity in the two lowest dosage groups (Fig. 6B). A similar tendency of the palmitoyl-CoA hydrolase activity was also observed in the peroxisomal-enriched and microsomal fractions (Fig. 6C and D). Noteworthy, BCMTD and CMTTD, as well as CETTD, feeding decreased the hydrolase activity in the peroxisomal-enriched and microsomal fractions (Fig. 6C and D).

Correlation between palmitoyl-CoA hydrolase and peroxisomal β-oxidation. Linear regression analysis of the individual cytosolic palmitoyl-CoA hydrolase and microsomal peroxisomal β-oxidation activities measured in this study indicated a correlation coefficient of $r = 0.94$ ($N = 18$, $P < 0.01$) (Fig. 7).

Morphological analyses. Peroxisomes. The proportion of cytoplasmic volume occupied by peroxisomes (Fig. 8A) and the total number of peroxisomes in hepatocytes (Fig. 8B) increased in a dose-dependent manner subsequent to both sulfur substituted dicarboxylic acid (BCMTD) and alkylthio acetic acid (CMTTD) feeding. The peroxisomes of BCMTD and CMTTD treated animals appear to be more heterogeneous in size, shape and matrix structure than those in hepatocytes of normal animals [25]. From Fig. 8B it can be seen that the average volume of peroxisomes increased in a dose-related fashion. It was interesting to note, however, that from the size distribution of peroxisomal particles [25] both BCMTD and CMTTD at 75 and 150 mg/day/kg body wt induced a marked polydispersity of liver peroxisomes, where populations of peroxisomes were both larger (Fig. 8C) and smaller (Fig. 8D) than normal peroxisomes. Moreover, the maximum induction of the volume fraction of peroxisomes (Fig. 8A), the average mean volume (Fig. 8B) and the size distribution of peroxisomal particles (Fig. 8C and D) occurred at different dose-levels for the two inducers. Based on these parameters BCMTD appears to be a much more potent peroxisome proliferator than CMTTD (3-6-fold), when considered at equal molar basis. Notably, the increase in volume fraction of peroxisomes in animals fed BCMTD and CMTTD at different doses (Fig. 8B) was similar in magnitude to the induced peroxisomal β-oxidation in the microsomal fraction (Fig. 5C) and not in the peroxisome-enriched fraction (Fig. 5B).

Alkylthio propionic acid (CETTD) increased the volume fraction of peroxisomes only 1.6-fold, mainly due to an increase in the size of the organelle (Fig.

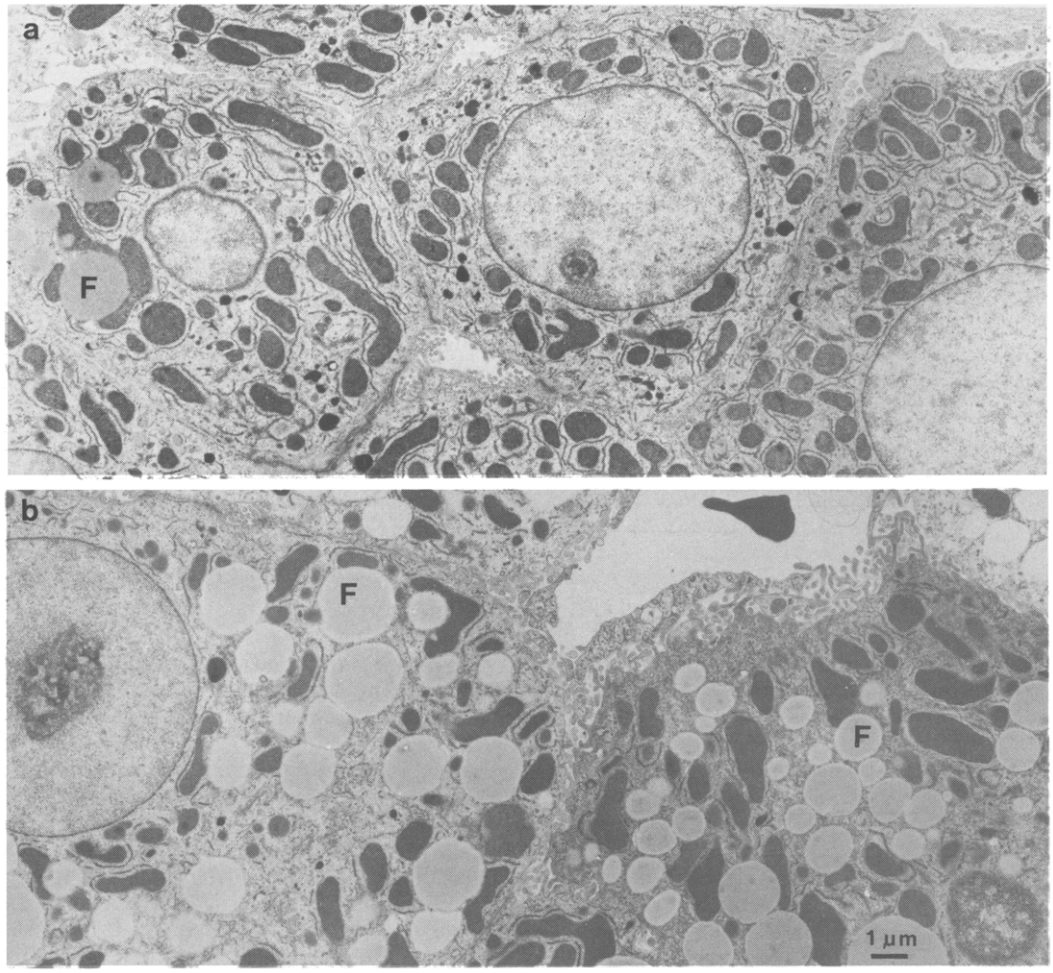

Fig. 9. Electron micrographs of the liver after treatment with carboxymethyl cellulose (control animals) (a) and 150 mg/day/kg body wt alkylthio propionic acid (b). F, fat-droplets.

8B and C). Thus, compared to the acids with a sulfur atom in the 3-position of carbon chain, CETTD showed almost no effect on peroxisome proliferation. The same phenomenon was observed after palmitic acid (Fig. 8) and hexadecanedioic acid adminstration (data not shown). Thus, the most dramatic effect of moving the sulfur atom from 3 to 4 position in the carbon chain of the sulfur substituted fatty acid analogues was development of fatty liver (Fig. 9).

Mitochondria. Subsequent to BCMTD treatment, the average mean volume of mitochondria (Fig. 10A) was decreased whereas the number of mitochondria was increased (Fig. 10B) in a dose-dependent manner. In contrast to BCMTD administration, in animals fed alkylthio acetic acids the mitochondria was enlarged, resulting in 1.5-fold increase of the mitochondria mean volume at a dose of 500 mg/day/kg body wt (Fig. 10A). Notably, at that dose, the number of mitochondria was almost normalized (Fig. 10B). Lower doses CMTTD increased the number of mitochondria about 1.5-fold (Fig. 10B). Alkylthio propionic acid (Fig. 10), palmitic acid and hexadecanedioic acid administration [25] only marginally effected the mitochondria morphology.

DISCUSSION

The present study has confirmed previous findings [25] that the size and number of peroxisomes and the hepatic peroxisomal β-oxidation capacity were markedly increased when male rats were fed non-β-oxidizable sulfur substituted fatty acid analogues, BCMTD and alkylthio acetic acid (CMTTD) (Figs. 5 and 8). Furthermore, administration of alkylthio propionic acid (CETTD), palmitic acid and hexadecandioic acid, acids which all are β-oxidizable, only marginally affected these hepatic responses (Figs. 5 and 8). However, our studies have shown that maximal induction of peroxisomal β-oxidation (Fig. 5) and proliferation of peroxisomes (Fig. 8) occurred at different dose levels for the three fatty acid analogues. Regarding the induction capacity the sulfur substituted dicarboxylic acid was apparently three to six times more potent than the mono acetic acid, when considered on a μmol/day basis.

Results presented here further showed that the sulfur substituted fatty acid analogues, especially the dicarboxylic acid and the alkylthio acetic acid, induced several mitochondrial and peroxisomal enzymes, both catabolic and anabolic, such as palmitoyl-CoA synthetase (mitochondria, peroxisomes,

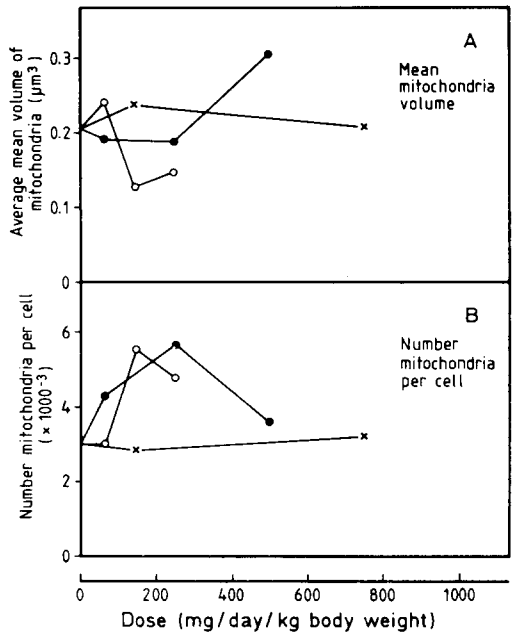

Fig. 10. Morphometric parameters of mitochondria in hepatocytes of rats fed increasing doses of BCMTD (○—○), CMTTD (●—●) and CETTD (×—×).

microsomes) (Fig. 2), palmitoyl-CoA hydrolase (mitochondria, peroxisomes, microsomes, cytosol) (Fig. 6), carnitine palmitoyltransferase (mitochondrial) (Fig. 3), peroxisomal β-oxidation (peroxisomes) (Fig. 5) and glycerophosphate acyltransferase (mitochondria, microsomes) (Fig. 4). Changes of these fatty acid metabolizing enzyme activities were not only revealed in total liver homogenates, but also at the subcellular level. Experimental studies in animals have shown that some ordinary long-chain fatty acids, particularly polyunsaturated fatty acids of fish origin, enhance fatty acid oxidation partly by increased peroxisomal activity and development of megamitochondria [12, 13]. Considering that the polyunsaturated long-chain fatty acids are relatively slowly metabolized, we found it likely that simple non-β-oxidizable fatty acid analogues might have similar effects.

Results presented here showed that the two sulfur substituted non-β-oxidizable fatty acid analogues, a dicarboxylic acid and a monoacetic acid do not only promote heterogeneous population of peroxisomes, resulting in larger and smaller organelles (Fig. 8) but they also induce proliferation of mitochondria (Fig. 10) especially with the alkylthio acetic acid (CMTTD). In general, increasing doses of BCMTD tended to decrease the volume fraction of mitochondria and this decrease appears to be a consequence of decreased size rather than a decrease in the number of mitochondria. Increasing doses of CMTTD, however, tended to normalize the number of mitochondria but in this case they were larger than in the controls [10]. For the later compound, this hepatic pleiotropic response is notably similar to those observed in animals treated with fish oil [12, 13, 31] which also contains a poorly metabolizable fatty acid.

The observed changes of mitochondria and peroxisomes may change the average sedimentation coefficient (s-value) for the organelles as already observed after clofibrate [2, 3], tiadenol [3] and high-fat feeding [12, 13]. Subsequently, changes of the recovery of mitochondria and peroxisomes in the mitochondrial-enriched and peroxisomal-enriched fractions may occur by the selected fractionation procedure. This effect has been verified after administration of the sulfur substituted fatty acid analogues. In the liver of normal rat most of the peroxisomal β-oxidation is revealed in the L-fraction. The subcellular distribution of the peroxisomal β-oxidation i.e., between the peroxisome-enriched (L) and microsomal (P) fractions was different in the three feeding groups (Fig. 5). Therefore, the 14-fold increase in recovery of specific peroxisomal β-oxidation activity in the P-fraction after dicarboxylic acid feeding compared to 30% and no increase after alkylthio acetic acid and alkylthio propionic acid, respectively (Fig. 5C), appears to be a consequence of a decrease in the s-value of the peroxisomes.

In the lowest BCMTD dosage group (75 mg/day/kg body wt) the mitochondrial carnitine palmitoyltransferase (sum of inner and outer CPT activity) increased more than two-fold as a result of BCMTD feeding (Fig. 3). Higher doses gradually decreased the carnitine palmitoyltransferase activity. Thus, the decreased carnitine palmitoyltransferase activity at the highest dosage of BCMTD can be accounted for by the decreased s-value for the mitochondria (Fig. 10), i.e. lowering the recovery of these organelles in the mitochondrial fraction obtained by the selected fractionation procedure. Furthermore, increased carnitine palmitoyltransferase activity by CMTTD administration might be due to larger mitochondria.

The same consideration should be taken into account regarding the variation of the mitochondrial glycerophosphate acyltransferase activity (Fig. 4) and the mitochondrial palmitoyl-CoA synthetase activity (Fig. 2) after alkylthio acid treatment. Concerning the last enzyme activity, alkylthio propionic acid, however, at the lowest dose increased the peroxisomal palmitoyl-CoA synthetase activity (Fig. 2). Thus a specific induction of peroxisomal acyl-CoA synthetase by these sulfur substituted fatty acid analogues cannot be excluded.

The present study has confirmed our previous findings [25] that the total palmitoyl-CoA hydrolase activity of rat liver increased in animals adminstered BCMTD and CMTTD (Fig. 6). Regarding BCMTD and CMTTD feeding, the subcellular fraction studies revealed that increased palmitoyl-CoA hydrolase activity was mainly attributed to an increase in mitochondria and especially cytosolic fractions (Fig. 6B and C). In contrast to an increased palmitoyl-CoA synthetase (Fig. 2) a decrease of the palmitoyl-CoA hydrolase activity was observed in the peroxisomal-enriched fraction (Fig. 6). Thus, the observed palmitoyl-CoA hydrolase activity in the cytosol does not seem to be a consequence of a decrease in the s-value at peroxisomes. The increase in cytosolic activity could rather be caused by a translocation of the microsomal enzyme [3, 4, 15]. BCMTD and

CMTTD administration revealed a close association between the numerical increases in peroxisomes (Fig. 8), the numerical increase of the specific palmitoyl-β-oxidation (Fig. 5), and the numerical increase of the cytosolic palmitoyl-CoA hydrolase activity (Figs. 6 and 7). Whether increased cytosolic palmitoyl-CoA hydrolase activity is a good indicator of peroxisome proliferation, should be considered [25].

Peroxisome proliferating agents has been shown to cause a transient hepatic lipid accumulation [32]. The data presented in this paper show that in contrast to BCMTD-induced peroxisomal β-oxidation which reach the maximum activity in the highest dosage group (500 mg/day/kg), including the mitochondrial glycerophosphate acyltransferase, the microsomal glycerophosphate acyltransferase activity was increased to its maximum value at a dose of 150 mg/day/kg (Fig. 4). One might expect that a 150 mg dose, giving an increased glycerophosphate acyltransferase activity, would give an increase in hepatic triglycerides. However, a dramatic reduction of triglycerides was observed (Table 2). A similar phenomenon has been observed after CMTTD treatment (data not published). In contrast, CETTD administration which only slightly affected the glycerophosphate acyltransferase activity (Fig. 4) increased the hepatic lipid dramatically (Fig. 9). These data show that increased glycerophosphate acyltransferase activity is not correlated to increased triglyceride content. Rather an inversed correlation was found with these peroxisome proliferating agents. BCMTD induced proliferation of peroxisomes and mitochondria (Figs. 8 and 10). All these effects would seem to require an increase in the rate of synthesis of membrane lipids, and hence, an increase in glycerophosphate acyltransferase activity.

Alkylthio propionic acid (CETTD) treatment gave a dramatically increased hepatic triglyceride accumulation and reduced the serum triglyceride pool (Table 2 and Fig. 9). The cyanide-sensitive palmitoyl-CoA oxidation in isolated mitochondria from CETTD-fed animals, was dramatically inhibited (over 70%) compared to fatty acid oxidation in mitochondria of control animals (data to be published). In contrast to alkylthio propionic acid, BCMTD treatments reduced, not only the serum triglyceride concentration, but also the hepatic triglyceride content (Table 2). Whether the lowering of plasma triglycerides by the sulfur substituted fatty acid analogues reflects diminished lipogenes, increased fatty acid oxidation, possible in peroxisomes and diminished secretion of triglyceride by the liver, requires further investigation.

In conclusion, the data suggest that induction of peroxisome proliferation and peroxisomal β-oxidation generally requires a sustained high level of the inducer i.e. long-chain acyl-CoA and/or xenobiotic CoA thioesters [24, 25]. The dietary treatment with ordinary fatty acids, palmitic acid and hexadecanedioic acid, only marginally affected the peroxisomal enzyme system, probably because the required threshold value was not exceeded. The same explanation can be offered for the metabolizable sulfur substituted fatty acid analogue, CETTD. It is expected that the concentration of the inducer of poorly metabolizable compound exceed the concentration of metabolizable compound on the basis of equal molar concentration. The order of the inducing agents with regard to their effects on the activities of key enzymes involved in fatty acid oxidation and esterification, including palmitoyl-CoA synthetase, peroxisomal β-oxdiation and peroxisome proliferation was BCMTD > CMTTD ≫ CETTD. The sulfur substituted fatty acid analogues are substrates for the acyl-CoA synthetase (data to be published). Whether accumulation of unmetabolizable xenobiotic CoA thioesters versus metabolizable CoA thioesters including long-chain acyl-CoA [17, 24, 25] is a property of all peroxisome proliferation should be considered. The level of CoA thioester derivatives by the compounds may determine their potency as peroxisomal proliferators.

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